



TITLE:

Sputum YKL-40 Levels and Pathophysiology of Asthma and Chronic Obstructive Pulmonary Disease.

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**SPUTUM YKL-40 LEVELS AND PATHOPHYSIOLOGY OF ASTHMA AND
CHRONIC OBSTRUCTIVE PULMONARY DISEASE**

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Running title: Sputum YKL-40 levels in asthma and COPD

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Conflicts of Interest: None

Abstract

Background: Recent evidence suggests that YKL-40, also called chitinase-3-like-1, is involved in the pathogenesis of asthma and chronic obstructive pulmonary disease (COPD).

Details of sputum YKL-40 in asthma and COPD, however, remain unknown.

Objectives: To clarify associations of sputum YKL-40 levels with clinical indices in asthma and COPD.

Methods: Thirty-nine patients with asthma, 14 age-matched never-smokers as controls, 45 patients with COPD, and seven age-matched smokers as controls. Sputum YKL-40 levels were measured and YKL-40 expression in sputum cells was evaluated by immunocytochemistry.

Results: Sputum YKL-40 levels were higher in patients with COPD (346 ± 325 ng/ml) than in their smoker controls (125 ± 122 ng/ml; $p < 0.05$), but were not significantly different between patients with asthma (117 ± 170 ng/ml) and their controls (94 ± 44 ng/ml; $p = 0.15$).

In patients with asthma only, sputum YKL-40 levels were positively correlated with disease severity ($r = 0.34$, $p = 0.034$) and negatively correlated with pre- and post-

bronchodilator %FEV₁ ($r = -0.47$ and -0.42 , respectively, $p < 0.01$) and forced mid-

expiratory flow ($r = -0.48$ and -0.46 , respectively, $p < 0.01$). Sputum YKL-40 levels were

positively correlated with sputum neutrophil counts in asthma ($r = 0.55$, $p < 0.001$) and with

neutrophil and macrophage counts in COPD ($r = 0.45$ and 0.65 , respectively, $p < 0.01$).

YKL-40 was expressed in the cytoplasm of sputum neutrophils and macrophages in all groups.

Conclusions: Elevated sputum YKL-40 reflects airflow obstruction in asthma, whereas the roles of YKL-40 in the proximal airways in COPD remain to be elucidated.

51 **Abbreviation list**

52 CHI3L1: chitinase-3-like-1

53 BALF: bronchoalveolar lavage fluid

54 COPD: chronic obstructive pulmonary disease

55 GOLD: the Global Initiative for Chronic Obstructive Lung Disease guidelines

56 FEF_{25-75%}: forced mid-expiratory flow

57 NE: neutrophil elastase

58 MBP: major basic protein

59 ICS: inhaled corticosteroids

60 IL: interleukin

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Introduction

YKL-40, also known as chitinase-3-like-1 protein (CHI3L1) or human cartilage glycoprotein-39, is classified as a “mammalian chitinase-like protein,” although it does not exhibit chitinase activity. YKL-40 is produced in response to inflammatory stimuli and is secreted by several types of cells, including neutrophils [1], macrophages [2], chondrocytes, synovial cells [3], and vascular smooth muscle cells [4]. YKL-40 exhibits a potent proliferative activity in skin and fetal lung fibroblasts [5] and stimulates the migration of vascular smooth muscle cells and vascular endothelial cells [4, 6]. Serum YKL-40 levels are elevated in patients with various diseases such as hepatic fibrosis, systemic sclerosis, osteoarthritis, and idiopathic pulmonary fibrosis [7], suggesting the involvement of YKL-40 in inflammatory processes and tissue remodeling [8].

Asthma and chronic obstructive pulmonary disease (COPD) are characterized by airway inflammation and remodeling that lead to reversible or irreversible airflow obstruction. Recent studies have shown that YKL-40 is involved in the pathophysiology of asthma [9-12] and COPD [13]. Serum YKL-40 levels were higher in patients with asthma [9] and COPD [13] than in healthy controls and were correlated with airflow obstruction and disease severity. Serum YKL-40 levels were higher in patients with asthma with exacerbations than those in a stable condition [11]. In patients with COPD, elevated YKL-40 levels in the bronchoalveolar lavage fluid (BALF) have been reported to be associated with airflow obstruction. In the case of asthma, Kuepper et al. showed that YKL-40 levels in the BALF of patients with allergic asthma were increased after administration of segmental allergen challenges [12]. However, associations of YKL-40 levels in the airways with clinical indices in asthma remain largely unknown.

Sputum YKL-40 levels may provide more relevant and specific information on asthma than that provided by levels found in blood samples. Therefore, we investigated the

88 relationships of sputum YKL-40 levels with clinical indices in asthma and assessed the
89 similarities and differences in sputumYKL-40 associations with disease pathophysiology
90 between asthma and COPD.

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Methods and Materials

Subjects

For this cross-sectional study, 39 patients with stable asthma who regularly visited our outpatient asthma and cough clinic were enrolled. Asthma was diagnosed according to the American Thoracic Society criteria [14] based on a history of recurrent episodes of wheezing and chest tightness, with or without cough, and documented airway reversibility with a bronchodilator or hyperresponsiveness to inhaled methacholine. Severity was defined according to the step classification of the Global Initiative for Asthma guidelines, as revised in 2002 [15], and classified as follows: mild intermittent (step 1), mild persistent (2), moderate persistent (3), and severe persistent (4). All patients with asthma were lifelong never-smokers.

Patients with COPD ($n = 45$) as defined by the Global Initiative for Chronic Obstructive Lung Disease guidelines (GOLD) 2003 [16] who had a history of chronic respiratory symptoms, such as cough and sputum with or without breathlessness and had a post-bronchodilator FEV₁/forced vital capacity (FVC) ratio of less than 0.7 and who regularly visited our outpatient COPD clinic were recruited. Patients were either current ($n = 10$; mean of 62.9 ± 26.3 pack-years) or former smokers ($n = 35$; 62.7 ± 28.8 pack-years). Typical emphysematous changes were observed in all patients with COPD on chest computed tomography scans. Among these, six were considered to have chronic bronchitis that was defined by the presence of sputum production for a consecutive 3 months for 2 years in a row. The conditions of both asthma and COPD patients were stable, and they had been free of exacerbations for 4 weeks or more. Patients were excluded who had any active malignant diseases within 5 years, connective tissue diseases, infectious diseases, or active respiratory disorders other than asthma or COPD.

We recruited 14 age-matched healthy never-smokers as controls for patients with

asthma and seven age-matched former smokers without COPD as controls for patients with COPD from our hospital. The research protocol was approved by the Ethics Committee of Kyoto University, and written informed consent was obtained from all subjects.

Sputum induction and processing

Sputum induction and processing were performed as described by Pin [17], with slight modifications [18]. In brief, the subjects were pre-medicated with inhaled salbutamol (200 µg). They then inhaled hypertonic (3%) saline solution, administered by an ultrasonic nebulizer (MU-32, Azwell Inc, Osaka, Japan) for 15 minutes. Adequate sputum plugs were separated from saliva and first treated with 0.1% dithiothreitol (Sputasol, Oxiod Ltd., Hampshire, UK), followed by the same volume of Dulbecco's phosphate buffered saline (PBS). After centrifugation, sputum supernatants were stored at -80°C. Cell differentials were determined by counting at least 400 non-squamous cells stained by the May-Grünwald-Giemsa method.

Measurement of YKL-40 levels in sputum supernatants

YKL-40 levels in sputum supernatants were measured using an enzyme-linked immunosorbent assay kit (Quidel, San Diego, USA) following the manufacturer's instructions. The detection limit of this assay was 10 ng/ml. Values below this threshold were assigned values of 10 ng/ml before adjusting for the dilution with dithiothreitol and PBS. A spike-back analysis that used exogenous YKL-40 resulted in greater than 80% recovery.

Specific IgE measurement

In patients with asthma and COPD, serum allergen-specific IgE antibodies were detected with a capsulated hydrophilic carrier polymer radioallergosorbent test fluoroenzyme

immunoassay (Phadia, Uppsala, Sweden) at an external laboratory (Mitsubishi Kagaku Bio-Clinical Laboratories, Kyoto, Japan), for mixed moulds, house-dust mite, cat dander, dog dander, Japanese cedar pollen, mixed grass pollens, and mixed weed pollens. Atopy was determined based on the detection of at least one allergen-specific IgE antibody.

Pulmonary function

We measured FVC, FEV₁, and forced mid-expiratory flow (FEF_{25-75%}) using a Chestac-65V (Chest MI Corp., Tokyo, Japan). Spirograms were obtained in triplicate, and the best of 3 reproducible measurements was recorded, as recommended by the American Thoracic Society/European Respiratory Society [19].

Immunostaining

Sputum cells from at least three samples obtained from patients with asthma, those with COPD and their age-matched controls were used for immunostaining. After adjusting for the cell number, sputum cells were mounted on slides by cytocentrifugation, air-dried, fixed in acetone/methanol (60:40), and stored at -20°C until immunostaining. For double immunostaining, samples were first blocked with 5% BSA in PBS for non-specific binding. The slides were then incubated either with a rabbit polyclonal antibody against human YKL-40 (33 µg/ml) (Quidel) or rabbit IgG (Dako, Glostrup, Denmark) at the same concentration as a control and either a monoclonal mouse antibody against human neutrophil elastase (NE) (Dako), CD68 (Dako), or major basic protein (MBP) (Chemicon, Temecula, CA, USA) or mouse IgG (Sigma-Aldrich, Tokyo, Japan) in PBS containing 1% BSA. Concentrations of mouse IgG used for negative controls are shown in Table 1. After rinsing in PBS, samples were incubated with Alexa Fluor 488 donkey anti-rabbit IgG (Invitrogen Corp, Carlsbad, CA, USA) and Alexa Fluor 546 goat anti-mouse IgG (Invitrogen). A fluorescence

169 microscope was used for immunocytochemical evaluations.

170 Positive staining was detected as green for the YKL-40 antigen and red for the NE,

171 CD68, and MBP antigens.

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173 **Statistical analysis**

174 A Mann-Whitney U-test was used to compare 2 groups. For comparisons of nominal data, a

175 chi-squared test or Fisher's exact test was used. Correlations were analyzed using

176 Spearman's rank correlation test. P-values of < 0.05 were considered significant. Differences

177 among 3 groups were first examined using a Kruskal-Wallis test. Results are given as means

178 \pm SDs, unless otherwise stated. Statistical analysis was performed using JMP 6.0 (SAS

179 Campus Drive, Cary, NC, USA).

RESULTS

Characteristics of patients with asthma and COPD and their age-matched controls

Characteristics, results of pulmonary function tests and sputum cell differentials of 39 patients with asthma and their age-matched controls are shown in Table 2. In the asthma group, differences in patient characteristics other than serum IgE levels between atopic (median serum IgE = 120 IU/ml) and non-atopic patients (median serum IgE = 39 IU/ml; $p = 0.037$) were not statistically significant. The findings for 45 patients with COPD and their age-matched smoker controls are shown in Table 3. Differences in patient characteristics between COPD patients with and without chronic bronchitis were not statistically significant. When patients with asthma and COPD were compared, patients with COPD were predominantly males and older than those with asthma, and more patients with asthma ($n = 38$) received inhaled corticosteroids (ICS) than did COPD patients ($n = 12$) ($p < 0.001$), and more patients with asthma used theophylline (9 patients with asthma vs 1 patient with COPD patient, $p = 0.005$). Patients with COPD showed severer airflow limitation (for FEV_1/FVC and $\%FEF_{25-75\%}$, $p < 0.001$; for $\%FEV_1$, $p = 0.042$) and showed greater number of macrophages and neutrophils in induced sputum ($p = 0.020$ and $p < 0.001$, respectively) than those with asthma.

Sputum YKL-40 levels in patient and control groups

Sputum YKL-40 levels were significantly higher in patients with COPD (346 ± 325 ng/ml) than in their smoker controls (125 ± 122 ng/ml; $p = 0.011$) (Fig 1a), whereas there was no significant difference between patients with asthma ($n = 39$, 117 ± 170 ng/ml) and their controls (94 ± 44 ng/ml) ($p = 0.15$). In 14 patients with asthma and two smoker controls, sputum YKL-40 levels were below the detection limit. Atopic status of patients with asthma did not affect sputum YKL-40 levels (atopic asthma 105 ± 125 ng/ml, non-atopic asthma 155

± 271 ng/ml; $p = 0.88$) (Fig 1b). For patients with COPD, differences in sputum YKL-40 levels between those who had chronic bronchitis ($n = 6$, 471 ± 384 ng/ml) and those who did not (327 ± 316 ng/ml; $p = 0.19$) were not statistically significant. When patients with asthma and COPD were compared, patients with COPD showed higher sputum YKL-40 levels than those with asthma ($p < 0.001$).

Relationships between YKL-40 sputum levels and clinical indices in patients with asthma and COPD

In patients with asthma, YKL-40 sputum levels were positively correlated with disease severity ($r = 0.34$, $p = 0.034$) (Fig 2) and maintenance doses of ICS ($r = 0.33$, $p = 0.045$), whereas in patients with COPD, there was no significant correlation between YKL-40 sputum levels and the GOLD stages ($r = -0.24$, $p = 0.11$) or maintenance doses of ICS ($r = 0.23$, $p = 0.13$). In patients with asthma, sputum YKL-40 levels were not associated with gender (males 148 ± 238 ng/ml, females 93 ± 74 ng/ml, $p = 0.88$). In either patient group, sputum YKL-40 did not associate with age (asthma, $r = -0.09$, $p = 0.50$; COPD, $r = 0.22$, $p = 0.15$). Moreover, body mass index, serum IgE levels, concurrent chronic sinusitis, and use of theophylline did not affect sputum YKL-40 levels (data not shown). In patients with asthma, sputum YKL-40 levels were negatively correlated with both pre- and post-bronchodilator FEV₁ (Fig 3A, B) and FEF_{25-75%} values (Fig 4A, B). In contrast, in COPD patients and the controls of both patient groups, no correlations were observed between YKL-40 sputum levels and measures of pulmonary function (Fig 3C, D and Fig 4C, D for COPD; data not shown for controls).

Relationships between YKL-40 sputum levels and sputum inflammatory cells:

Immunocytochemical examinations of sputum inflammatory cells

In patients with asthma, sputum YKL-40 levels were correlated only with the numbers of sputum neutrophils, while in COPD patients, sputum YKL-40 levels were correlated with the numbers of sputum macrophages and neutrophils (Fig 5). When the 14 patients with asthma who showed sputum YKL-40 levels under the detection limit were compared to the remaining 25 patients with asthma, the former showed fewer sputum neutrophils ($10.6 \pm 29.9 \times 10^5 \cdot g^{-1}$), in addition to higher pre-bronchodilator FEV₁ ($97.0 \pm 20.1\%$) and FEF_{25-75%} ($61.9 \pm 26.5\%$) values than the remaining 25 patients with asthma ($12.8 \pm 17.7 \times 10^5 \cdot g^{-1}$; $82.3 \pm 22.6\%$; $46.6 \pm 30.8\%$, respectively; $p < 0.05$ for all comparisons). No significant correlations were observed between sputum YKL-40 levels and sputum eosinophil counts in patients with asthma or in patients with COPD ($r = 0.12$, $p = 0.47$; $r = 0.25$, $p = 0.10$, respectively). There were no significant correlations between sputum YKL-40 levels and neutrophil, macrophage or eosinophil counts in the controls of both patient groups (data not shown).

The presence or absence of YKL-40 in CD68- or NE- positive cells in at least three samples obtained from patients with asthma, those with COPD, and their controls was examined immunocytochemically. The presence or absence of YKL-40 in MBP-positive cells was examined in patients with asthma. In all examined subjects, YKL-40 was positive for cells that were positive for NE or CD68 antigens, but was negative for cells that were positive for MBP (Fig 6). There were no apparent qualitative differences in the expression of YKL-40 in neutrophils or macrophages between patients with asthma and COPD and their controls. Apparent effects of age, gender and medications on YKL-40 expression were absent.

DISCUSSION

To our knowledge, this is the first study to examine sputum YKL-40 levels in patients with asthma and COPD. Sputum YKL-40 levels were elevated in patients with COPD compared with their age-matched smoker controls but did not differ between patients with asthma and their age-matched controls. In patients with asthma, sputum YKL-40 levels were positively correlated with disease severity and sputum neutrophil counts and were negatively correlated with measures of pulmonary function. In patients with COPD, no significant associations were found, except for those of sputum YKL-40 levels with macrophage and neutrophil counts.

Recent evidence suggests that chitinases [20] and chitinase like proteins including YKL-40 [21] are involved in the pathophysiology of asthma. Chupp et al. reported that serum YKL-40 levels were increased in patients with asthma and that these levels were positively correlated with disease severity (p value for trend = 0.02) and sub-basement membrane thickness in bronchial biopsy ($r = 0.51$, $p = 0.003$) and were negatively, although weakly, correlated with the levels of FEV₁ ($r = -0.22$, $p = 0.01$) [9]. The same researchers showed that the *CHI3L1* gene encoding YKL-40 had a single nucleotide polymorphism in its promoter region that was associated with elevated YKL-40 protein levels, asthma susceptibility, airway hyperresponsiveness, and impaired lung function [10]. Our results have confirmed and expanded upon previous findings.

In agreement with the results of the serum sample analysis performed by Chupp et al. [9], sputum YKL-40 levels in patients with asthma correlated with disease severity and degree of airflow obstruction. To evaluate irreversible functional changes, we recruited patients who were in a stable condition and also evaluated post-bronchodilator indices of pulmonary function. Moreover, unlike previous studies, we examined sputum samples; these samples provide more direct information on airway conditions than that provided by serum

samples. Consequently, correlations of YKL-40 levels with both pre- and post-FEV₁ values, as well as FEF_{25–75%} values, were stronger than those reported in a previous study on asthma [9]; this showed that YKL-40 in the airways was associated with airway remodelling in asthma.

Although the biologic functions of YKL-40 have not been completely understood, YKL-40 may be involved in persistent airway inflammation as well as tissue repair in asthma, as described below. Within 10 min after administration of segmental allergen challenges, the YKL-40 levels in BALF samples obtained from patients with allergic asthma increased and remained elevated for up to 24 h [12]. YKL-40 is induced by the pro-inflammatory cytokines tumour necrosis factor- α and interleukin (IL)-1 [22], as well as by IL-13 [23], which is a potential key regulator of asthma [24], and COPD [25]. Lee et al. showed that mice with null mutations of BRP-39 (BRP-39^{-/-}), a mouse homologue of YKL-40, showed markedly diminished antigen-induced Th2 responses and decrease in the ability of IL-13 to induce tissue inflammation and fibrosis [23]. YKL-40 also binds to collagen I and regulates collagen fibril formation [26]. These findings indicate potential biologic roles played by YKL-40 in airway inflammation and tissue remodelling in asthma.

In all groups, YKL-40 was expressed in the cytoplasm of sputum neutrophils, as well as macrophages. This finding was consistent with previous findings that showed the presence of YKL-40 in neutrophils and macrophages in BALF samples obtained from patients with COPD [13] and those with severe asthma [9]. We found new associations of sputum YKL-40 levels with sputum cell types. Sputum YKL-40 levels were correlated with sputum neutrophil counts in patients with asthma and with both macrophage and neutrophil counts in patients with COPD. In addition, patients with asthma who showed sputum YKL-40 levels below the detection limit revealed lower sputum neutrophil counts than the remaining asthmatic patients. This association of sputum YKL-40 levels with neutrophil

counts and expression of YKL-40 in sputum neutrophils suggests that neutrophils are the major cell source of sputum YKL-40 in asthma; this may partly explain the lack of a difference in sputum YKL-40 levels between asthmatic patients and their age-matched controls and the fact that a significant number of patients with asthma showed sputum YKL-40 levels below the detection limit. Neutrophilic airway inflammation plays an important role in a subgroup of patients with asthma [27] and is correlated with fixed airflow obstruction [28, 29] but is not a predominant feature in the patient population as a whole. In fact, sputum neutrophil counts were similar between patients with asthma and their age-matched controls in our study. Although correlations do not imply causation, in the case of asthma, YKL-40 in the airways may contribute to airflow obstruction in association with neutrophilic inflammation.

Recently, Tang et al. reported a moderate negative correlation of serum YKL-40 levels with %FEV₁ ($r = -0.44$, $p = 0.001$) and a mild correlation with peripheral blood eosinophil percentages ($r = 0.27$, $p = 0.032$) in patients with asthma [11]. In addition, Kuepper et al. reported that YKL-40 levels in BALF of patients with allergic asthma after administration of segmental allergen challenges were positively correlated with eosinophil counts in the BALF [12]. In our study, sputum YKL-40 levels were not correlated with sputum eosinophil counts. Our results, however, do not contradict previous findings because the asthmatic patients in our study were in a stable condition, and 74% of the patients in Tang's study had exacerbation attacks [11]. Moreover, neutrophilic and eosinophilic airway inflammation are not reciprocally exclusive in asthma, particularly in patients with worse asthma control [30].

In our study, the atopic status of patients with asthma did not affect sputum YKL-40 levels. Association studies of the *CHI3L1* gene with atopy have shown inconsistent findings; some single nucleotide polymorphisms were associated with risks for atopy [31], whereas

others showed protective effects [32]. Possible associations of YKL-40 with atopy should be further clarified.

Unexpectedly, we found no correlations between sputum YKL-40 levels and clinical indices, including the presence of chronic bronchitis, in patients with COPD. This finding was in contrast to the findings of Létuve et al., who reported negative correlations of BALF YKL-40 levels with FEV₁ values and carbon monoxide diffusion capacity in patients with COPD. They showed that YKL-40 contributed to the synthesis of pro-inflammatory and fibrogenic chemokines by alveolar macrophages in COPD [13]. The discrepancies between our findings and the findings of Létuve et al. cannot be fully explained but may be attributed to better %FEV₁ values in our study than in the study of Létuve et al. (the median %FEV₁ was 78.8% in our study and 61.5% in theirs) [13] and different sample sources, i.e., sputum vs. BALF. Sputum is derived from more proximal airways and contains fewer macrophages than those present in BALF. In addition to the lack of differences in sputum YKL-40 levels between COPD patients with and without chronic bronchitis, the discrepancy between our findings in COPD and asthma may suggest that YKL-40 in the airways is differently involved in the pathogenesis of COPD and asthma in terms of the locations, in particular, that are predominantly involved, although the findings in asthma and COPD in this study cannot be compared directly because there were significant differences in patients' characteristics such as age and gender between the two patient groups.

Our study has several limitations. First, the number of age-matched smoker controls was small because older smokers without airflow limitation were difficult to find and recruit. However, the difference in sputum YKL-40 levels between patients with COPD and smoker controls was significant. Second, we did not assess possible relationships between clinical indices and the degrees of YKL-40 expression in sputum cells because cells obtained from sputum samples were inadequate for quantifying the extent of YKL-40 expression. The

number of epithelial cells, which also express YKL-40 in severe asthma [9], was not assessed because the epithelial cells in the sputum were too few to be analyzed. Last, assigning values of 10 ng/ml when sputum YKL-40 levels were below this threshold may have overestimated actual sputum YKL-40 levels. Our findings, however, did not alter even when the values were assigned to 0.1 ng/ml (data not shown). The associations of sputum YKL-40 levels with clinical indices in asthma were robust.

In conclusion, elevated sputum YKL-40 levels reflect airflow obstruction only in asthma. Further analysis may be required to determine the roles of YKL-40 in the proximal airways in COPD.

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470

Figure legends

Figure 1. (a) Sputum YKL-40 levels between patients with COPD and their age-matched smoker controls. (b) Sputum YKL-40 levels between patients with atopic asthma and non-atopic asthma and their age-matched controls. $p = 0.34$ by Kruskal Wallis test. Logarithmic results are shown for sputum YKL-40 levels. Horizontal bars indicate mean values.

Figure 2. Correlation of sputum YKL-40 levels with disease severity in asthma ($r = 0.34$, $p = 0.034$).

Figure 3. Correlations of sputum YKL-40 levels with pre- and post-bronchodilator %FEV₁ in patients with asthma (A, B) and COPD (C, D).

Figure 4. Correlations of sputum YKL-40 levels with pre- and post-bronchodilator %FEF_{25-75%} in patients with asthma (A, B) and COPD (C, D).

Figure 5. Correlations of sputum YKL-40 levels with sputum neutrophil counts (A, C) and macrophage counts (B, D) in patients with asthma (A, B) and COPD (C, D).

Logarithmic results are given for sputum YKL-40 levels, and sputum neutrophil and macrophage counts.

Figure 6. Representative micrographs of sputum cytospin preparations. At least three samples obtained from patients and their age-matched controls are presented (Fig 6-1, healthy controls; Fig 6-2 and -4, atopic asthma; Fig 6-3 and -4, non-atopic asthma; Fig 6-5, smoker controls; Fig 6-6, COPD). M = male, F = female.

496 Double stained (a) with antibody against CD68 and negative control using rabbit IgG; (b)
497 with antibodies against CD68 and YKL-40; (c) with antibody against neutrophil elastase
498 (NE) and negative control using rabbit IgG; (d) with antibodies against NE and YKL-40; (e)
499 with negative control using mouse IgG and rabbit IgG; (f) with antibody against major basic
500 protein (MBP) and negative control using rabbit IgG; (g) with antibodies against MBP and
501 YKL-40.
502 Red indicates NE, CD68, and MBP; green indicates YKL-40; orange results for merged
503 images.
504

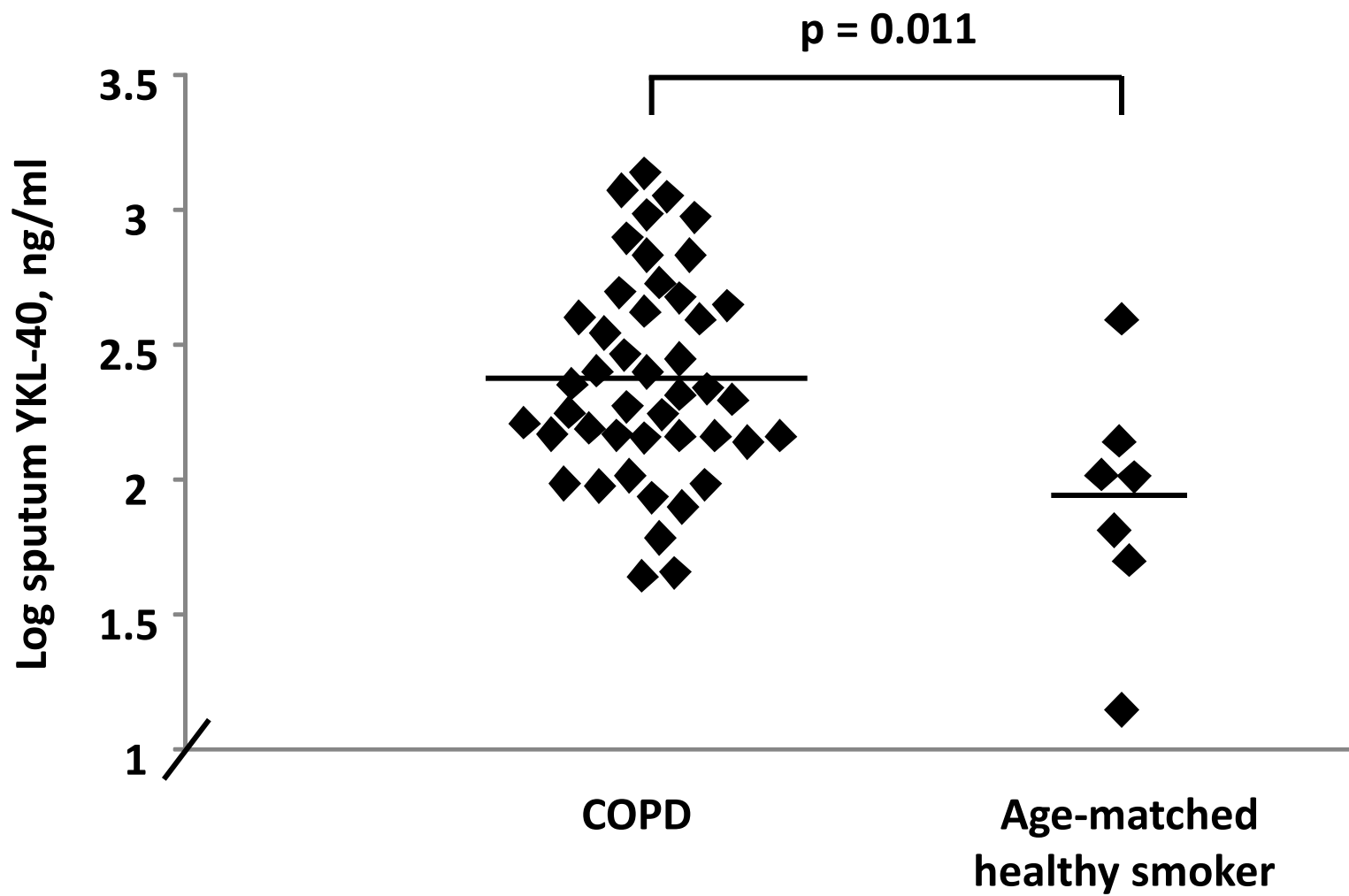


Fig. 1a

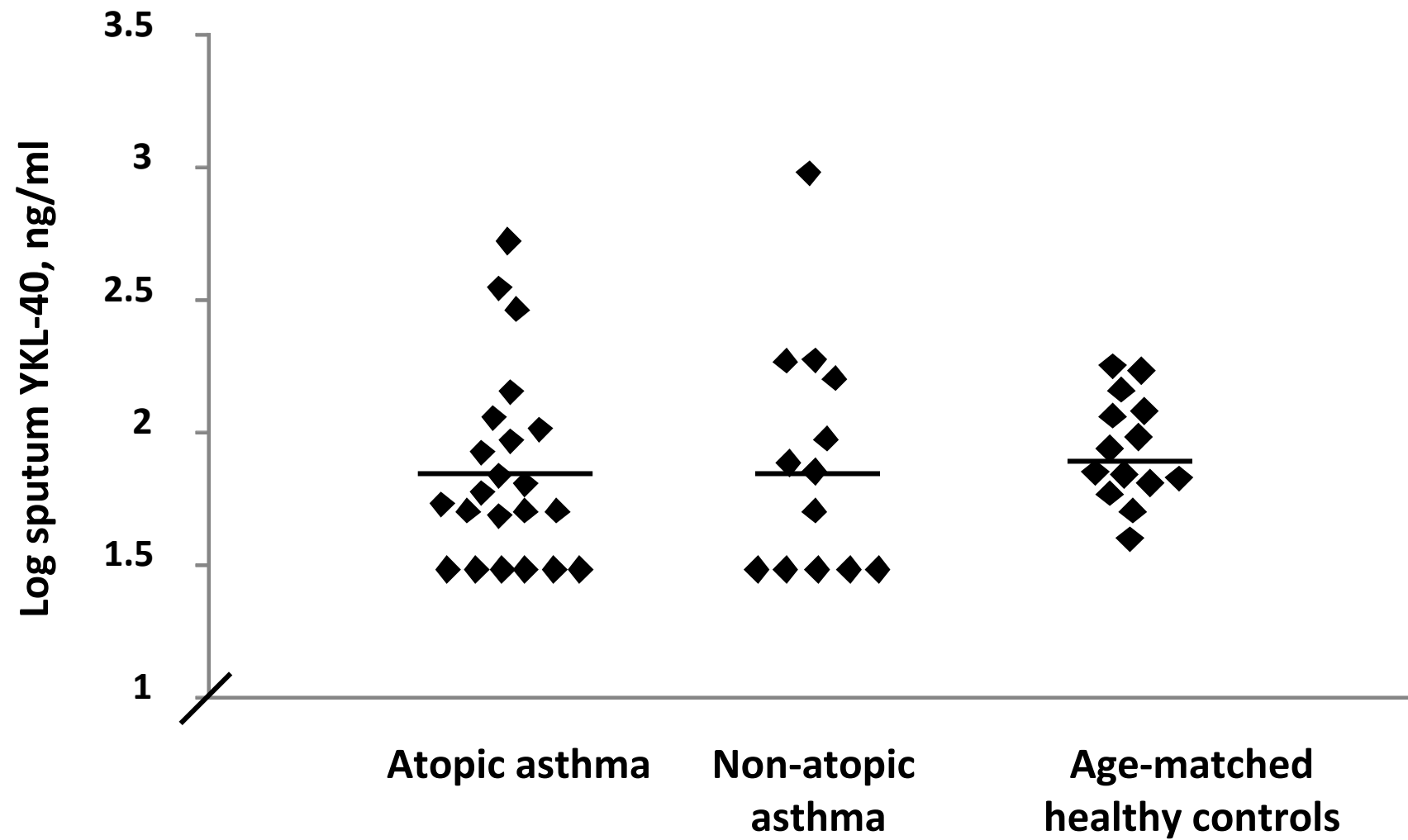


Fig. 1b



Fig. 2

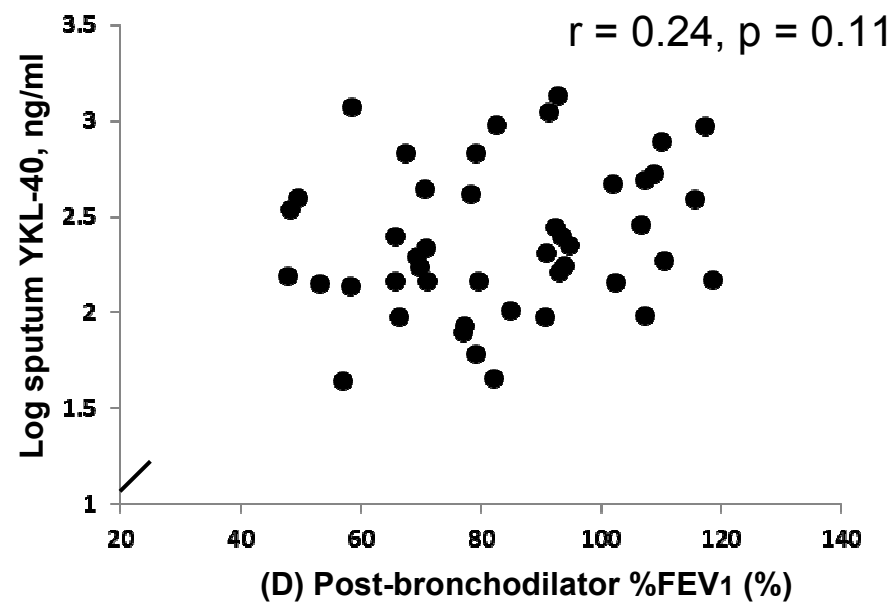
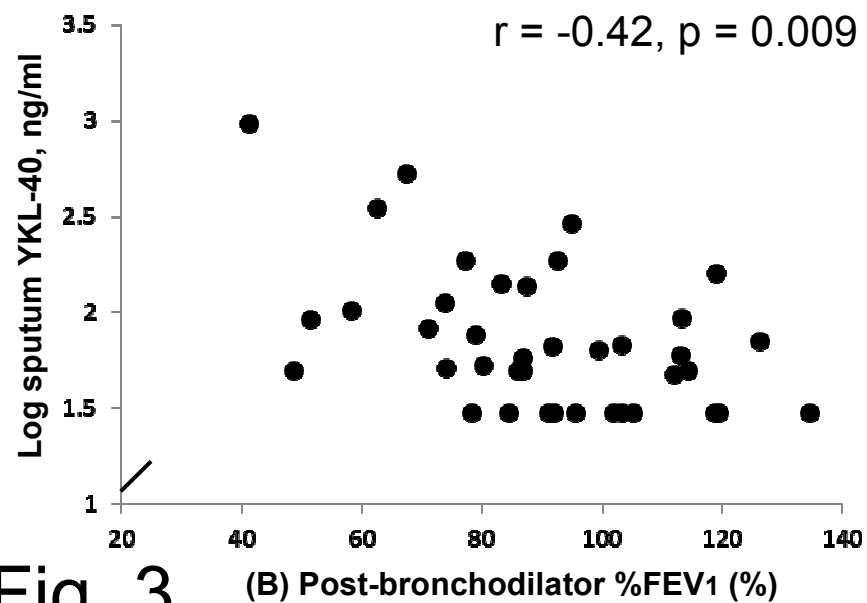
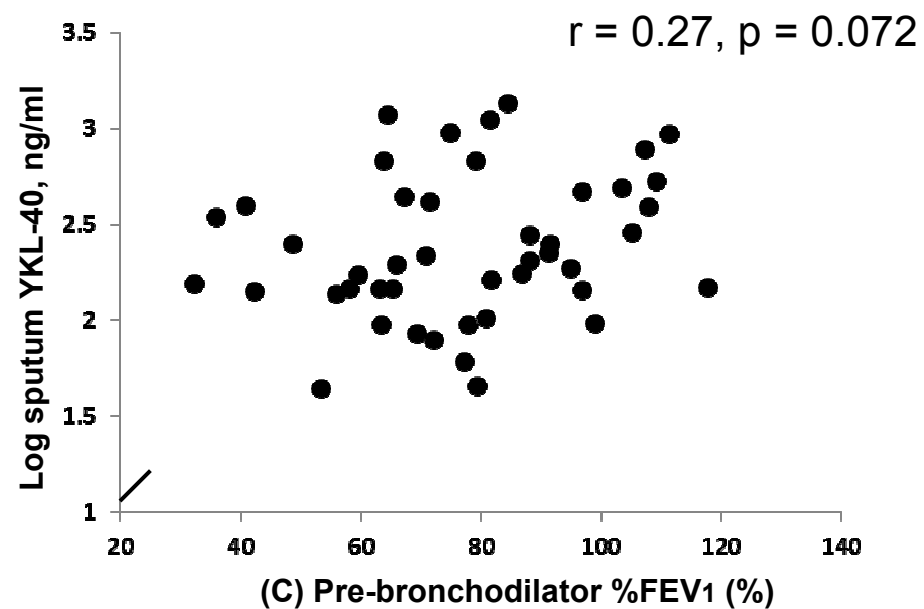
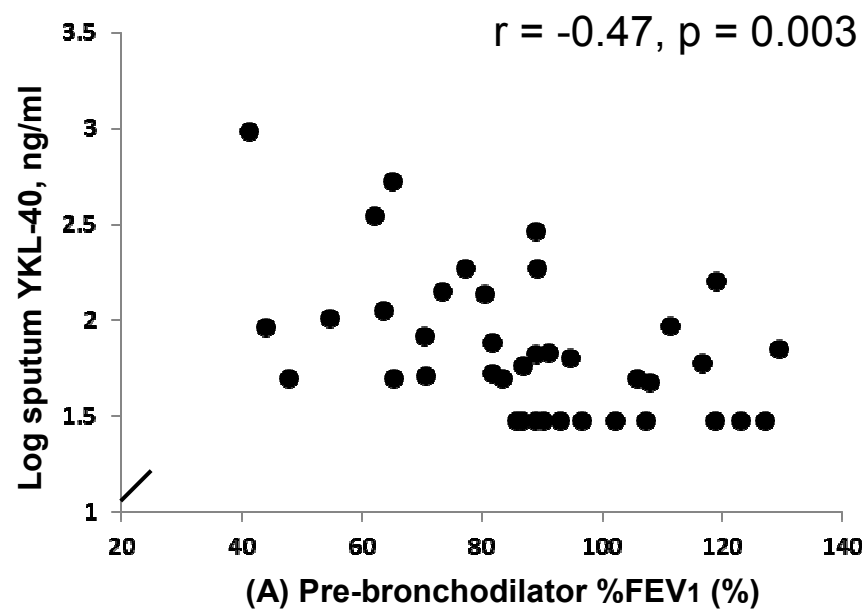


Fig. 3

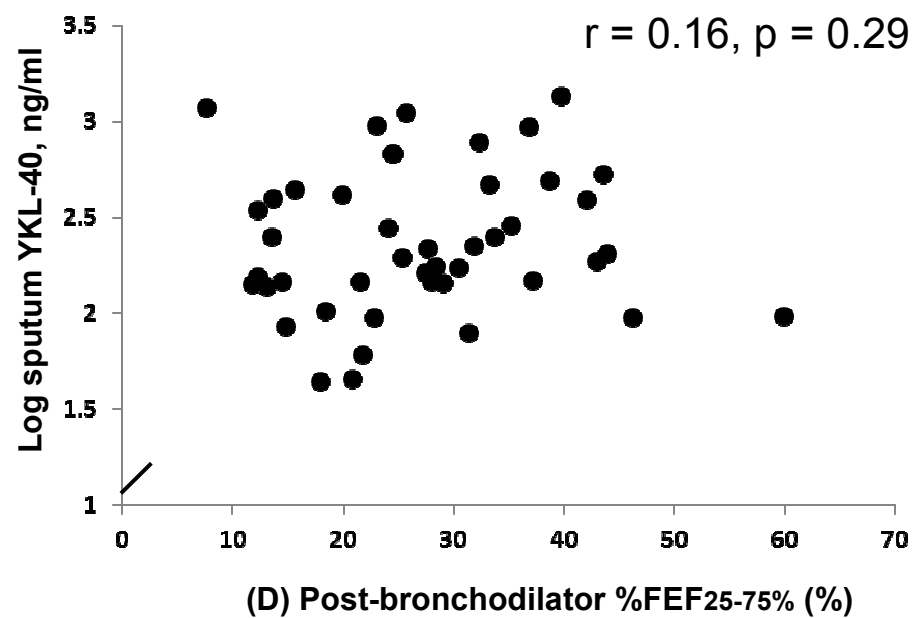
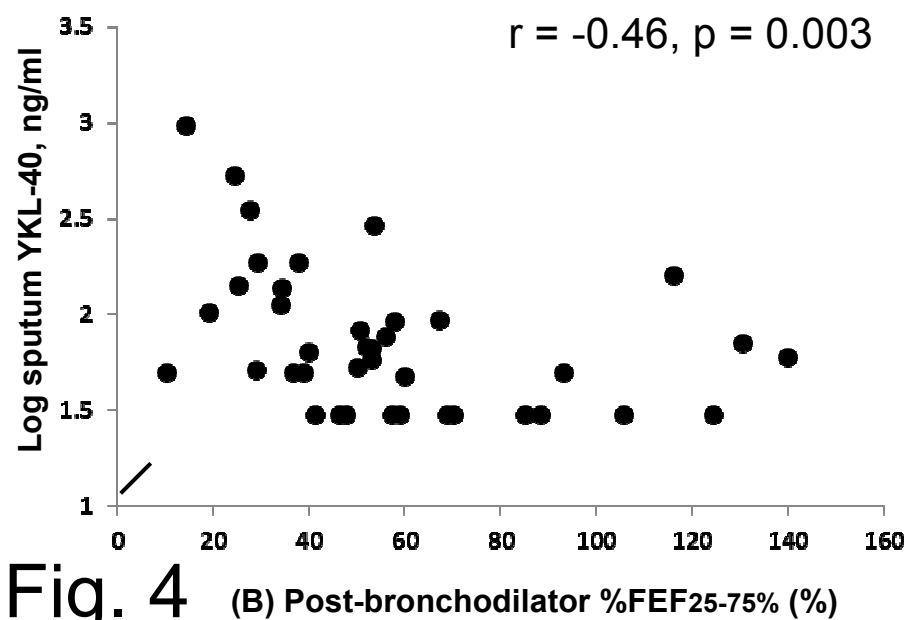
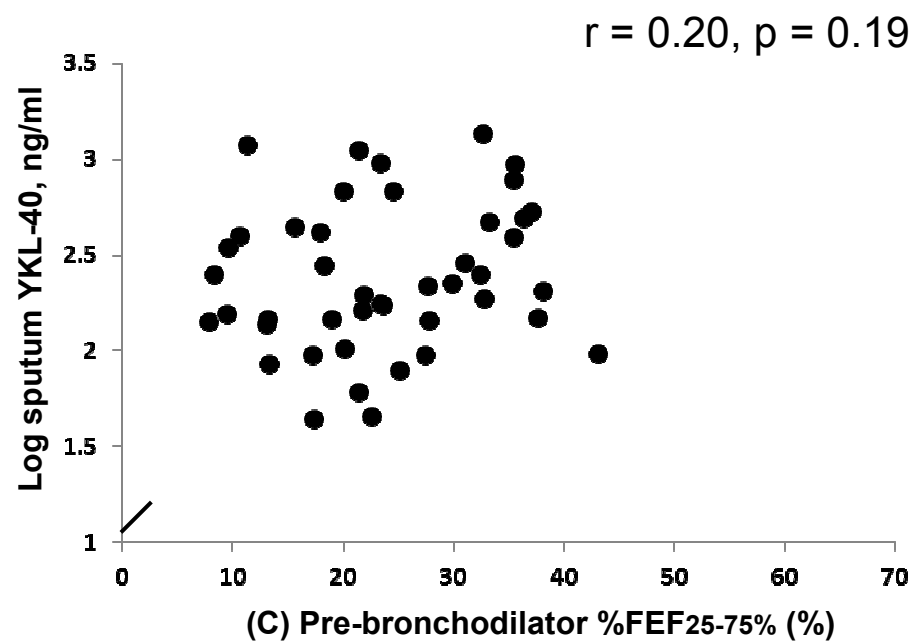
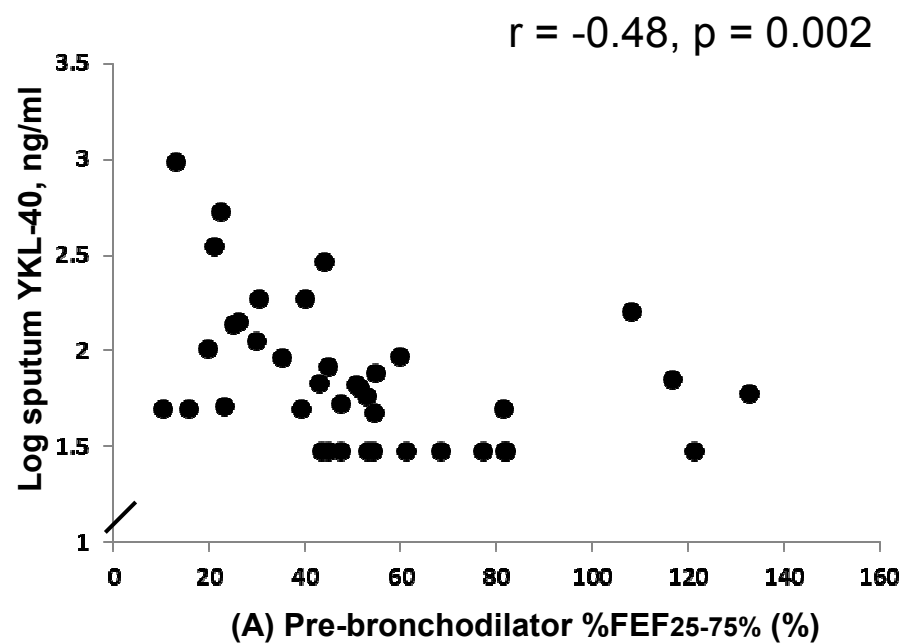


Fig. 4

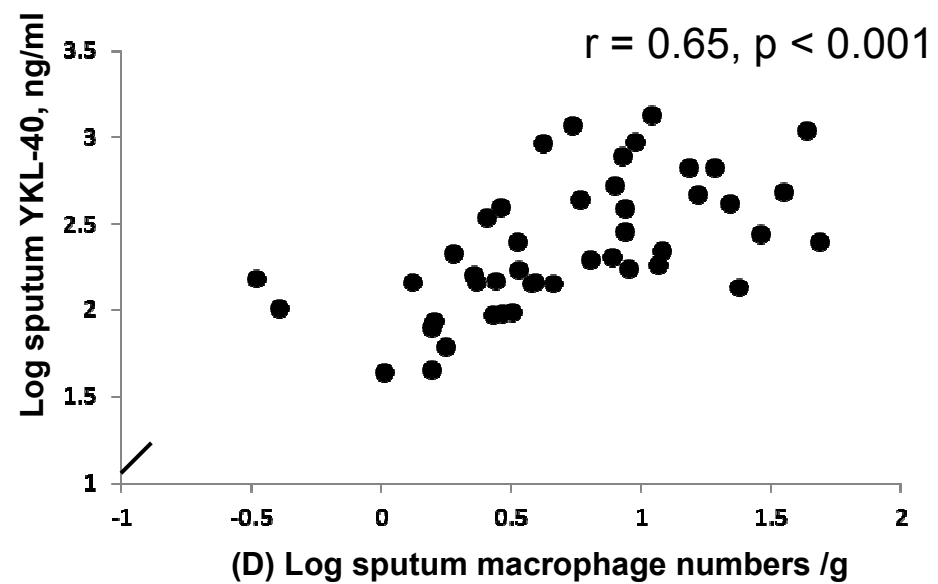
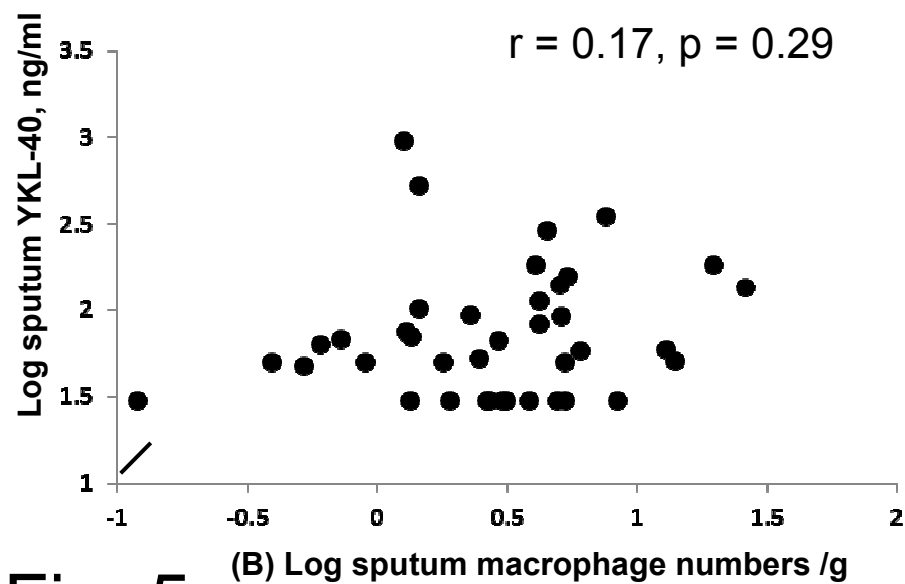
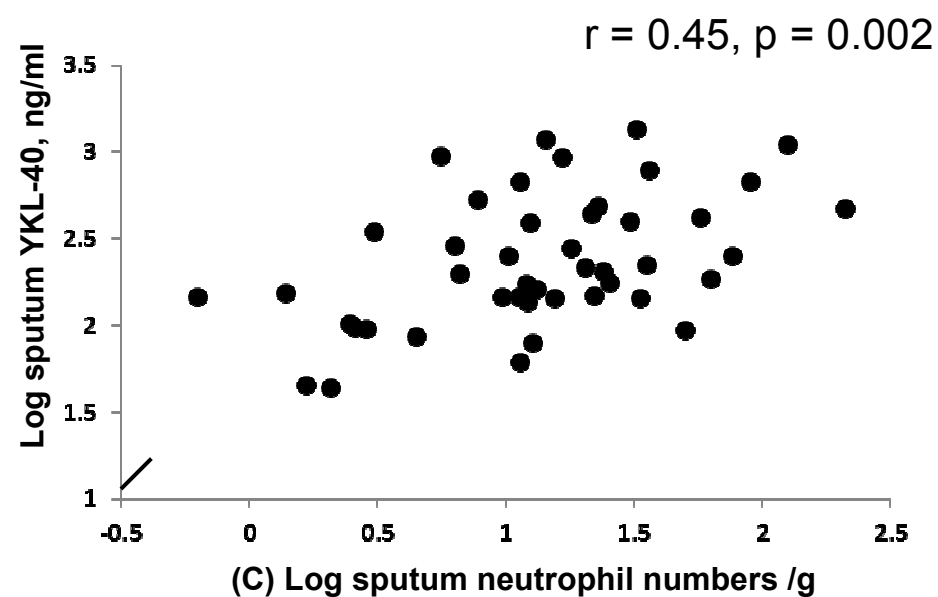
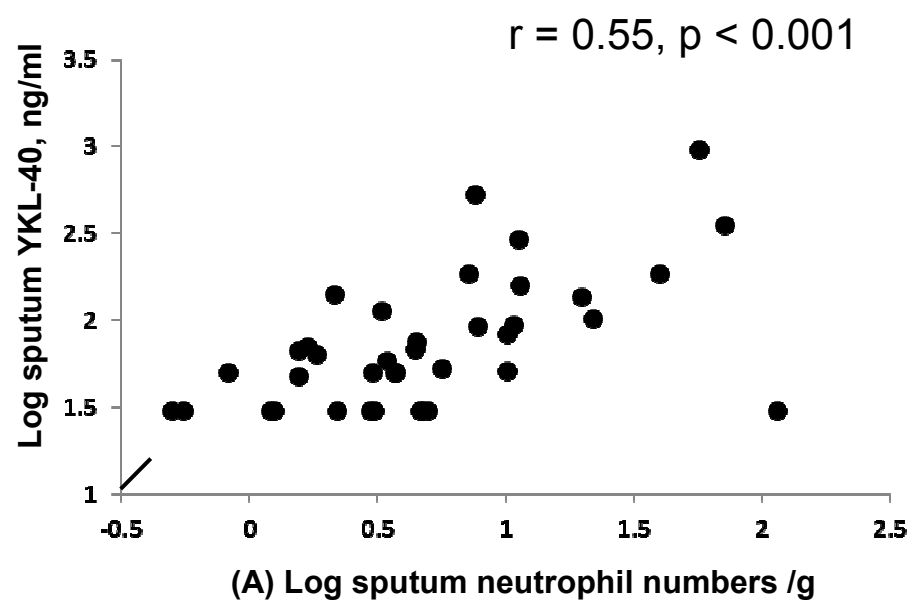


Fig. 5

Healthy controls

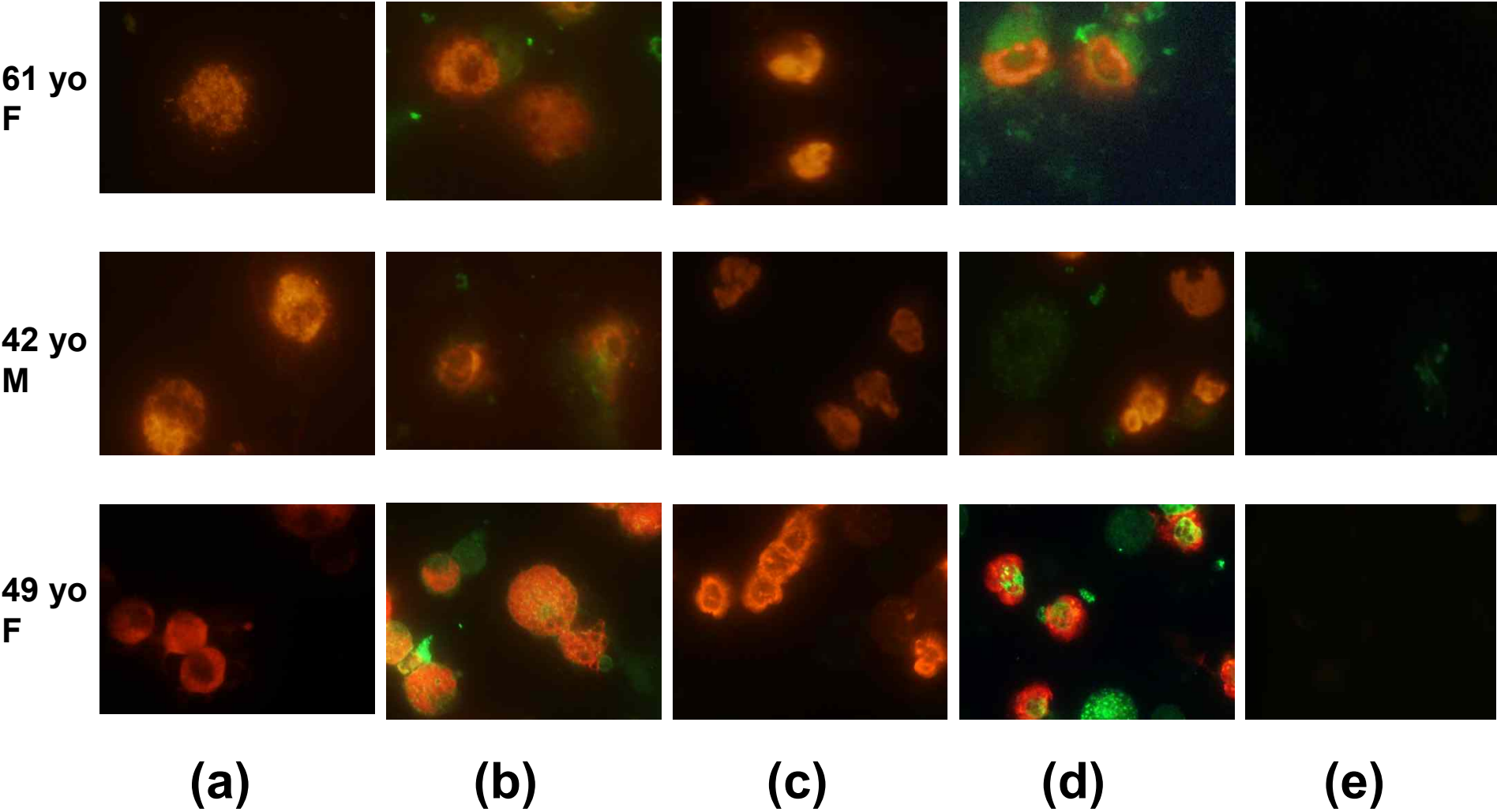


Fig. 6-1

Atopic asthma

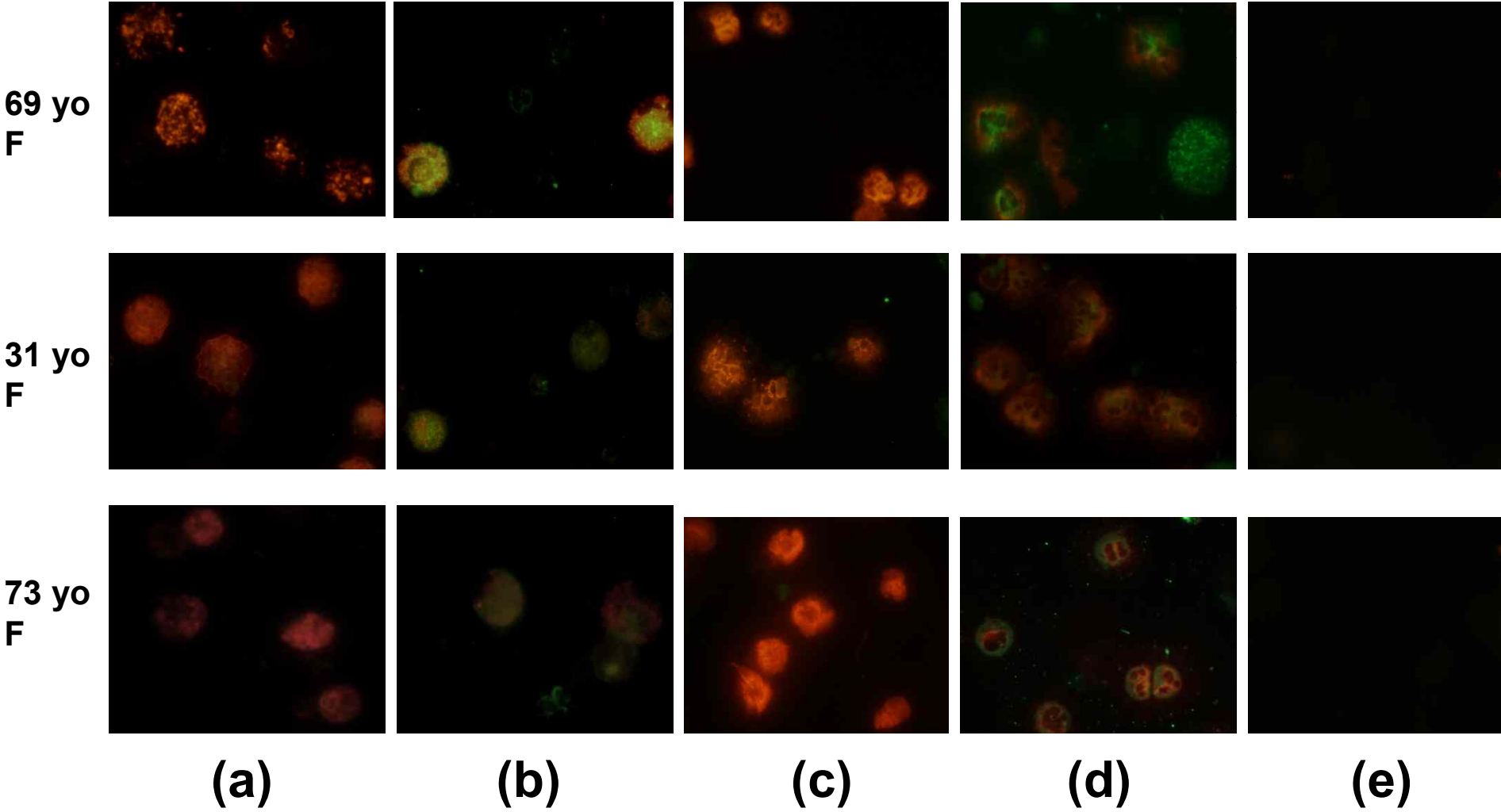
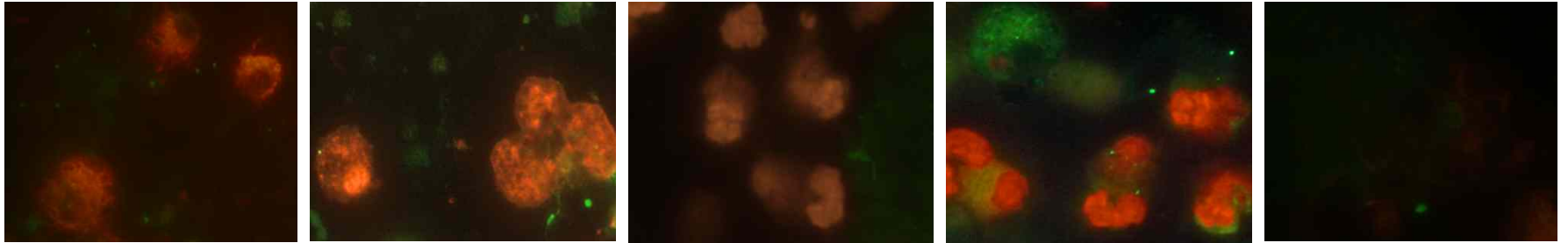


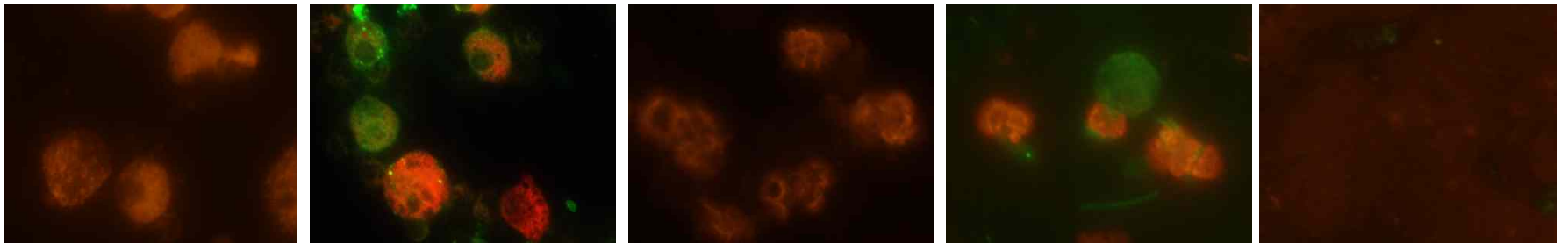
Fig. 6-2

Non-atopic asthma

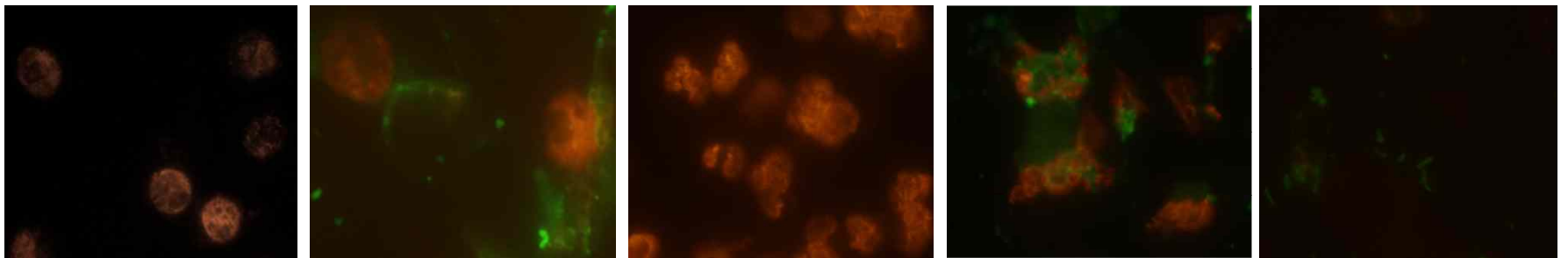
75 yo
F



36 yo
F



30 yo
F



(a)

(b)

(c)

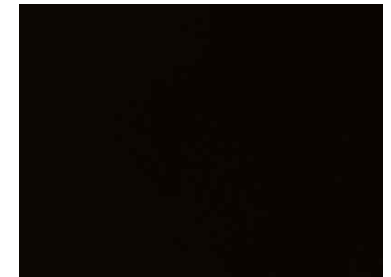
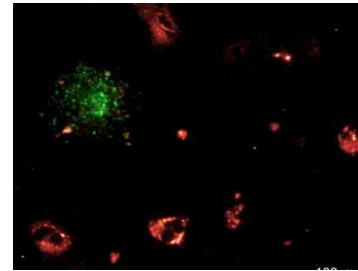
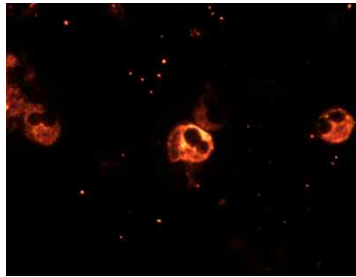
(d)

(e)

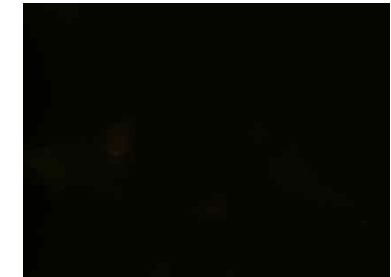
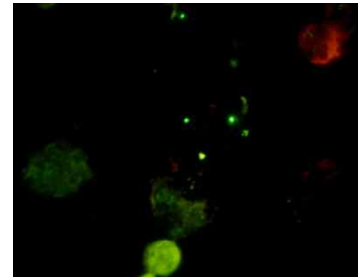
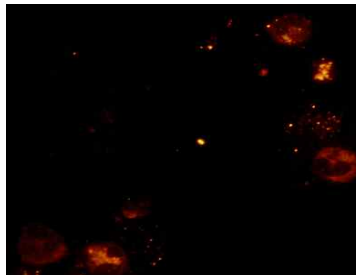
Fig. 6-3

Atopic asthma

70 yo
F

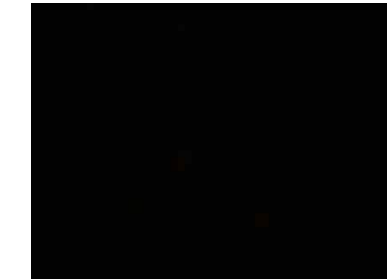
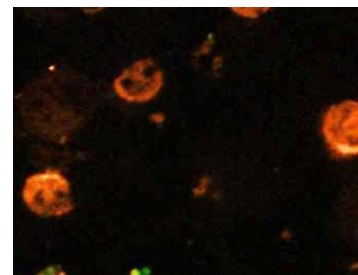
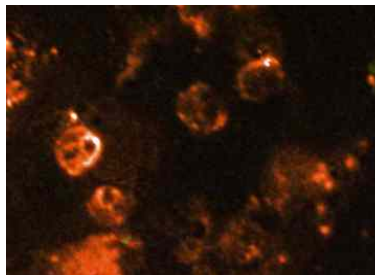


73 yo
F

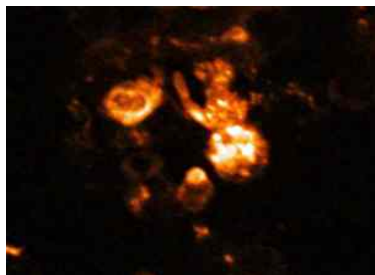


Non-atopic asthma

66 yo
M



66 yo
M



(f)

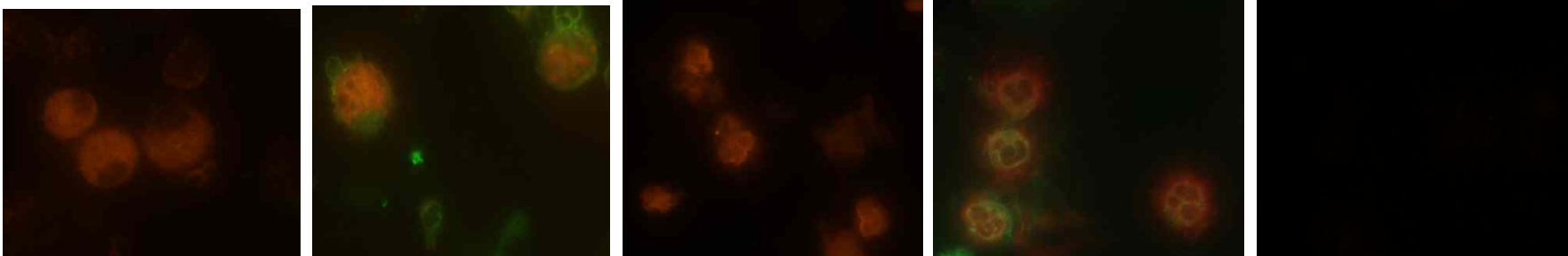
(g)

(e)

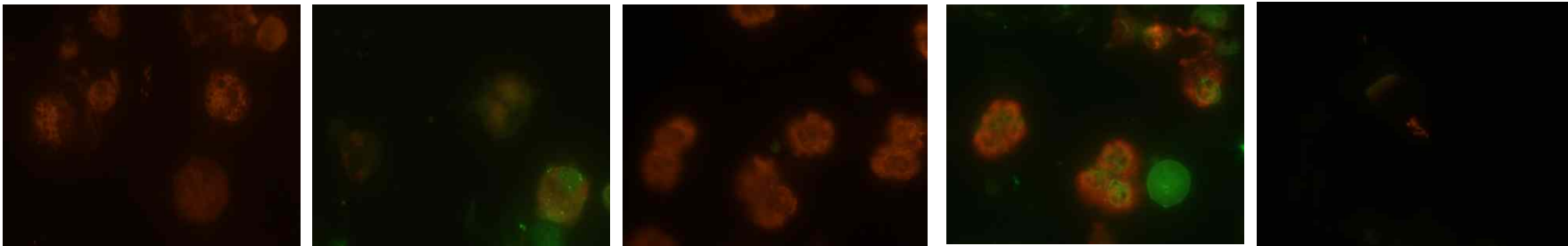
Fig. 6-4

Smoker controls

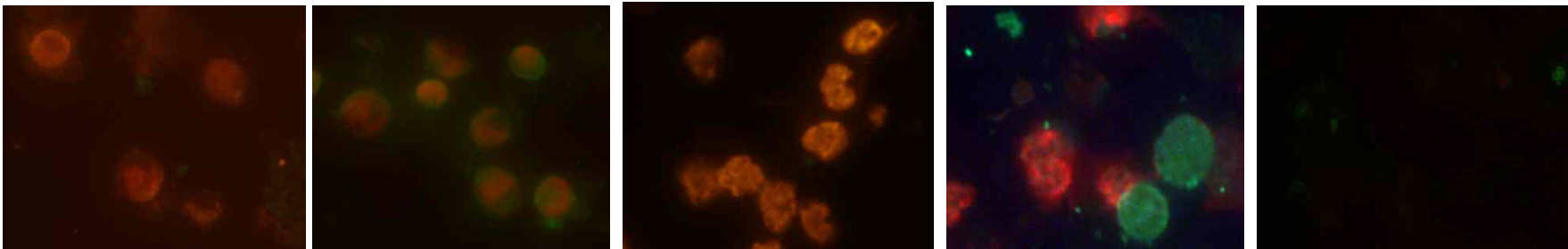
**74 yo
M**



**69 yo
M**



**63 yo
M**



(a)

(b)

(c)

(d)

(e)

Fig. 6-5

COPD

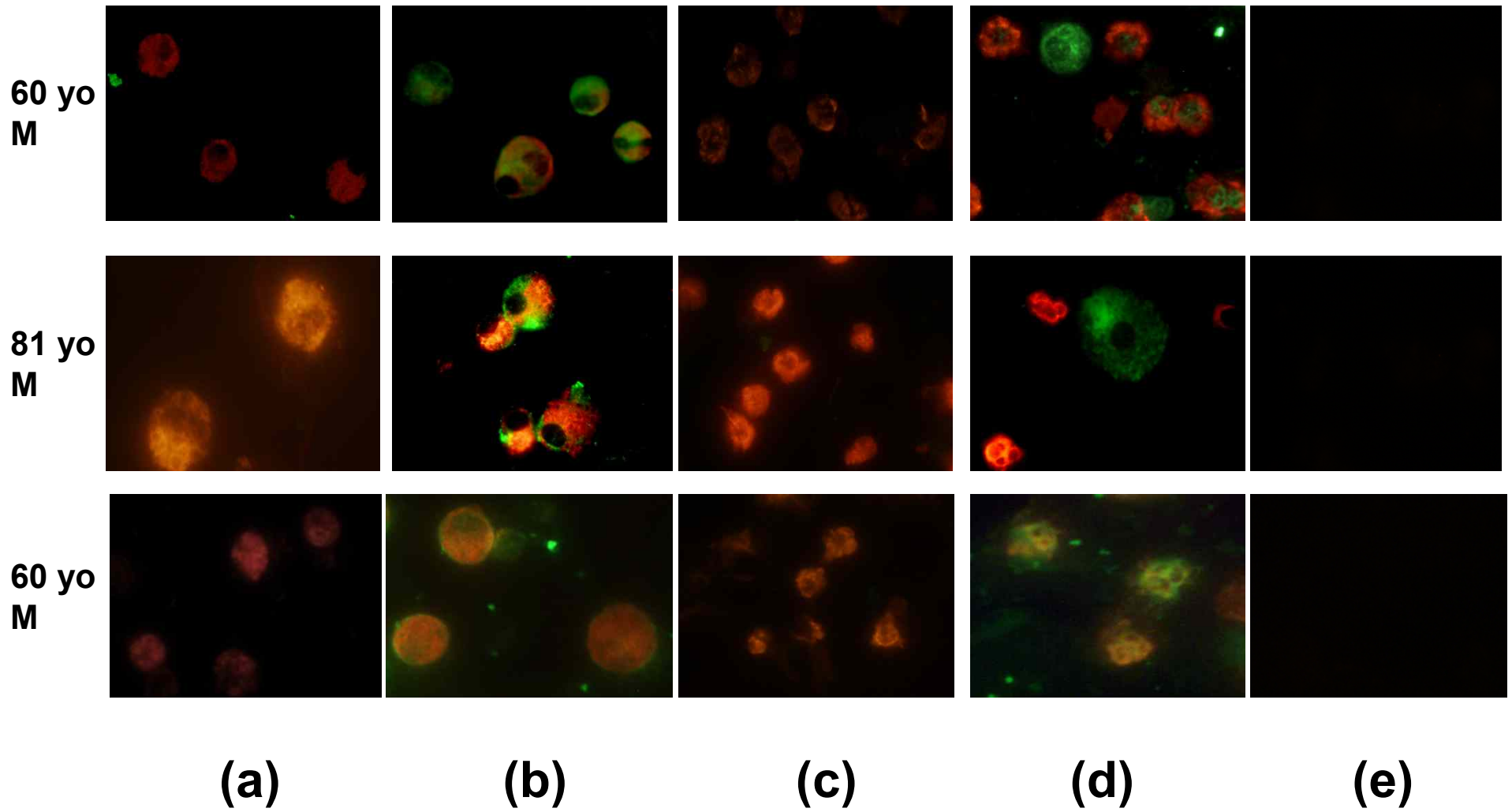


Fig. 6-6

505 Table 1. Concentrations of mouse IgG used for negative controls

Mouse monoclonal antibody	Concentration of mouse IgG
Anti-neutrophil elastase	0.07 µg/ml
Anti-CD68	1.25 µg/ml

506

507

Table 2. Characteristics and findings of patients with asthma and their age-matched controls

	Age-matched		p value
	Asthma N = 39	Control N = 14	
Male, n	12	5	0.75
Age, yrs	58 (14)	51 (14)	0.47
Body mass index, kg/m ²	23.7 (3.3)	22.0 (2.7)	0.083
Disease duration (years)	16.3 (17.2)	-	-
Disease severity *	1/17/12/9	-	-
Co-morbidity of chronic sinusitis	7	-	-
Atopy †, n	22	-	-
Serum IgE, IU/ml	118 (8-1276)	-	-
Dose of inhaled steroids, µg/day ‡	897 (607)	-	-
Pre-bronchodilator			
FEV ₁ /FVC, %	67.9 (11.8)	81.3 (5.9)	< 0.001
FEV ₁ , % predicted	87.6 (22.6)	105.8 (12.6)	0.003
FEF _{25-75%} , %predicted	52.1 (29.9)	93.3 (27.9)	< 0.001
Post-bronchodilator			
FEV ₁ /FVC, %	69.5 (11.6)	82.2 (6.3)	< 0.001
FEV ₁ , % predicted	90.3 (21.9)	108.4 (12.6)	0.006
FEF _{25-75%} , %predicted	57.3 (32.1)	96.7 (31.6)	< 0.001
Induced Sputum			
Macrophages × 10 ⁵ · g ⁻¹	4.6 (5.3)	6.4 (7.3)	0.76
Neutrophils × 10 ⁵ · g ⁻¹	12.0 (22.5)	6.6 (4.5)	0.55
Eosinophils × 10 ⁵ · g ⁻¹	2.6 (6.4)	0.1 (0.3)	< 0.001

Results are means (SD) except for IgE, median (range).

*Step classification of the Global Initiative for Asthma (1/2/3/4)

†Data are missing for 4 patients with asthma.

‡Dose equivalent to chlorofluorocarbon beclomethasone. The dose for patients untreated with inhaled corticosteroids was assigned 0 µg/day.

Table 3. Characteristics and findings of patients with COPD and their age-matched smoker controls

	COPD N = 45	Age-matched smoker control N = 7	p value
Male, n	45	6	0.13
Age, yrs	72 (9)	67 (5)	0.13
Body mass index, kg/m ²	22.0 (2.7)	22.6 (2.3)	0.82
Lifetime smoking, former: current	35: 10	5: 2	0.66
Pack-years	62.7 (27.9)	36.3 (12.8)	0.004
Disease duration (years)	6.2 (5.2)	-	-
Disease severity *	20/20/5/0	-	-
Co-morbidity of chronic sinusitis	4	-	-
Atopy, n	14	-	-
Serum IgE, IU/ml	130 (5-1500)	-	-
Dose of inhaled steroids, µg/day ‡	364 (637)	-	-
Pre-bronchodilator			
FEV ₁ /FVC, %	51.5 (10.7)	73.8 (9.1)	<0.001
FEV ₁ , % predicted	77.3 (21.1)	96.1 (8.6)	0.018
FEF ₂₅₋₇₅ %, %predicted	23.9 (9.3)	65.3 (22.7)	<0.001
Post-bronchodilator			
FEV ₁ /FVC, %	52.9 (11.3)	76.7 (7.4)	<0.001
FEV ₁ , % predicted	83.4 (19.9)	100.2 (8.1)	0.033
FEF ₂₅₋₇₅ %, %predicted	27.1 (11.3)	74.0 (17.4)	<0.001
Induced Sputum			
Macrophages × 10 ⁵ · g ⁻¹	9.4 (11.3)	2.9 (3.2)	0.033
Neutrophils × 10 ⁵ · g ⁻¹	26.9 (38.0)	8.8 (8.7)	0.046
Eosinophils × 10 ⁵ · g ⁻¹	1.5 (2.6)	0.5 (0.6)	0.13

Results are means (SD) except for IgE, median (range).

*Stages of GOLD criteria stages for COPD (I / II / III / IV)

‡Dose equivalent to chlorofluorocarbon beclomethasone. The dose for patients untreated with inhaled corticosteroids was assigned 0 µg/day.